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Survival of the functional yeast *Kluyveromyces marxianus* B0399 in fermented milk with added sorbic acid

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ABSTRACT

In this study, the survival of the functional yeast *Kluyveromyces marxianus* B0399 in an industrially produced fermented milk was evaluated. In particular, the yeast viability was assessed throughout the entire shelf-life of the product (30 d) to ensure the presence of the effective yeast dose (20 million viable cells for each serving of 125 g) while avoiding, by sorbic acid addition, yeast growth, which could affect product quality and stability. To find the best combination of yeast and sorbic acid concentration, 13 different combinations were tested, and then 2 of them were chosen for industrial production. In production at lower concentrations (30 million viable cells, 150 mg/kg of sorbic acid) the effective dose was maintained only at 4 and 6°C, whereas at higher dosages (70 million viable cells, 250 mg/kg of sorbic acid) the effect of temperature was less evident. In all the trials, the concentration of sorbic acid was not affected by microbial metabolism and remained stable throughout the entire shelf-life.
Key words: functional yeast, fermented milk, sorbic acid, *Kluyveromyces marxianus*

INTRODUCTION

Probiotics are defined as viable microorganisms that, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2002). After ingestion, they must overcome biological barriers (such as gastric acidity and bile toxicity) to reach the gastrointestinal tract and exert their health-promoting effects (Sánchez et al., 2009). Moreover, before their addition in functional foods, their safety and efficacy have to be demonstrated. Foods used as probiotic sources are mainly dairy products, such as yogurt, fermented milk, and cheese, are regarded as ideal vehicles for delivering probiotic microorganisms to the human gastrointestinal tract (Ross et al., 2002). Most of the microorganisms employed as probiotics are bacteria belonging to the genera *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Bifidobacterium*, *Enterococcus*, and *Bacillus* (Shah, 2004). Recently, an increased interest in probiotic fungi has been observed. *Saccharomyces boulardii* has, for a long time, been the only yeast commercialized as a probiotic for humans (Martins et al., 2005) and its beneficial effects have been demonstrated (Surawicz et al., 1989; Mombelli and Gismondo, 2000). In recent years much research has been focused on the isolation and characterization of yeasts from a technological and functional point of view (Pennacchia et al., 2008; Binetti et al., 2013). Among yeasts, *Kluyveromyces marxianus* (formerly *Kluyveromyces fragilis*), isolated from different dairy products (Farnworth, 2005; Bolla et al., 2011; Tofalo et al., 2014), has gained attention because of its peculiar characteristics (i.e., high thermotolerance and growth rate, broad substrate spectrum, and absence of fermentative metabolism upon sugar excess). Regarding its biotechnological applications, *K. marxianus* is well known for the production of enzymes (Fonseca et al., 2008), of aromatic compounds (Fabre et al., 1995), and ethanol in high-temperature processes (Fonseca et al., 2008). Moreover, it can be used also for the reduction of lactose content (Rajoka et al., 2004), for the recovery of high-value bioingredients (Belem et al., 1997). Finally, many studies reported its use for heterologous gene expression (Dellomonaco et al., 2007; Raimondi et al., 2008, 2013).

On the contrary, little information is available on the putative functional properties of *K. marxianus* (Yoshida et al., 2005; Romanin et al., 2010). Recently, Maccaferri et al. (2012a) investigated the potential of the strain *K. marxianus* B0399, isolated from milk, for its application as a probiotic. This strain was chosen because it is

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included in the European Food Safety Authority list of qualified presumption of safety biological agents added to food and feed (EFSA Panel on Biological Hazards, 2010). Moreover, it is able to survive the gastrointestinal transit, maintaining its vitality and fermentation capacity (Mustacchi et al., 2010). Studies of this strain have shown that it is able to affect colonic microbiota, increasing the bifidobacterial concentration in the colonic model system, and to induce the formation of higher amounts of the short-chain carboxylic acids acetate and propionate. Moreover, it is highly adhesive to human enterocyte-like Caco-2 cells and can modulate the immune response (Maccaferri et al., 2012a). These findings demonstrated that *K. marxianus* B0399 possesses several beneficial and strain-specific properties that make it suitable for application as a probiotic. Hence, some studies were focused on the effect of consumption of a fermented milk containing *Bifidobacterium animalis* ssp. *lactis* BB12 and *K. marxianus* B0399 on patients with irritable bowel syndrome (Lisotti et al., 2011; Maccaferri et al., 2012b).

Together with its functional potential, the technological properties of the strain *K. marxianus* B0399 need to be better investigated. In particular, a good viability throughout the entire shelf-life of the product in which it is incorporated is essential to induce the claimed benefits and, therefore, to commercialize the product as a functional food. In fact, a minimum daily intake of probiotics has to be assured to exert its beneficial functions (Vinderola et al., 2000).

The aim of this work was to optimize the formulation of a fermented milk containing *K. marxianus* B0399 to maintain at least 20 million viable yeast cells for each serving (a 125-g cup) throughout 30 d of refrigerated storage. This yeast amount was defined by studies evidencing beneficial effects with dosages between 10 and 20 million viable cells (Mustacchi et al., 2010; Lisotti et al., 2011; Maccaferri et al., 2012a). However, in this specific case, the viability of *K. marxianus* B0399 had to be maintained while avoiding its growth, which could affect product quality and stability (ethanol and CO_2 production, off-flavors, and so on). Therefore, sorbic acid was added to the formulation to prevent yeast multiplication. Thus, the main purpose of this research was to find the best combination of yeast concentration and sorbic acid (added at concentrations far below amounts able to exert a lethal effect on *K. marxianus* B0399), able to ensure a cell load of at least 20 million viable cells without product alteration. Moreover, a fast chromatographic method for the determination of sorbic acid in the samples was set up modifying the method proposed by other authors (Kamankesh et al., 2013).

MATERIALS AND METHODS

Kluyveromyces marxianus B0399 Strain

The probiotic strain *K. marxianus* B0399 was provided by Turval Laboratories (Udine, Italy) as a concentrated cell suspension in a liquid medium. The functional properties of this strain were reported by Lisotti et al. (2011), Maccaferri et al. (2012a,b), and Mustacchi et al. (2010).

To evaluate the effect of sorbic acid on *K. marxianus* B0399, MIC and minimum fungicidal concentrations (**MFC**) of this compound were assessed on yeast extract peptone-dextrose agar (**YPD**; Oxoid, Basingstoke, UK) at pH 6.5 (normal pH of the medium) and 4.2 (YPD added with proper amounts of HCl, 1 *N*), to simulate the pH of the tested product. For this determination, 198 μL of YPD inoculated with the strain at a level of approximately 5 log cfu/mL were placed into 200-μL microtiter wells (Corning Inc., Corning, NY). Because of the scarce solubility in water, an alcoholic stock solution (100 g/L) of sorbic acid (Fluka, Milan, Italy) was prepared and further diluted in ethanol. Two microliters of the proper dilutions were added to each well to convey different amounts of sorbic acid, obtaining the same final concentration of ethanol (1% vol/vol).

The sorbic acid concentrations tested ranged from 0 to 1,000 μg/mL for trial at pH 6.5 and from 0 to 500 μg/mL for trial at pH 4.2. A control with ethanol alone was also included. For each sorbic acid concentration, 8 repetitions were considered. Microtiter plates were incubated at 37°C and, for each condition, the presence of a visible growth after 72 h of incubation was recorded. The MIC was defined as the lowest concentration of the compound preventing visible growth of the inoculated cells after 72 h in all the 8 repetitions; MFC was defined as the lowest concentration of sorbic acid that caused the death of the inoculated cells, as no growth was observed (for all the 8 repetitions) after 72 h of incubation at 37°C of a 10-μL spot plated onto YPD agar.

Fermented Milk Production

Fermented milk used in our study was industrially produced from raw cow milk (4,000 L) that was concentrated, homogenized, and pasteurized. Then, milk was cooled, inoculated with *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and fermentation was carried out at 42°C until the pH reached 4.3 (about 7 h), after which the yogurt was stirred. At the end of fermentation, pomegranate puree (25% wt/wt) and *Bifidobacterium animalis* ssp. *lactis* BB12 (1%

Table 1. Experimental plan adopted to find the best combination of *Kluyveromyces marxianus* B0399 and sorbic acid able to satisfy company requirements (at least 20 million viable cells per serving throughout the entire shelf-life) avoiding package swelling (experimental plan section)[1]

	Experimental plan			Results after 30 d of storage at 6°C		
	Cell load of *K. marxianus*			Cell load of *K. marxianus*		
Combination	Cell number/serving	Log cfu/g	Sorbic acid (mg/kg)	(log cfu/g)	Cell survival (%)	Sorbic acid (mg/kg)
1	30 million	5.38	150	5.37 (±0.08)	97.64	154.62 (±2.82)
2	50 million	5.60	150	5.58 (±0.03)	95.49	159.80 (±3.88)
3	70 million	5.75	150	5.73 (±0.05)	95.57	152.20 (±3.76)
4	30 million	5.38	200	5.18 (±0.03)	63.33	196.36 (±5.95)
5	50 million	5.60	200	5.25 (±0.04)	45.03	204.45 (±4.56)
6	70 million	5.75	200	5.45 (±0.17)	53.34	209.66 (±2.89)
7	30 million	5.38	250	5.14 (±0.05)	57.99	251.08 (±3.18)
8	50 million	5.60	250	5.29 (±0.10)	49.65	259.42 (±6.76)
9	70 million	5.75	250	5.43 (±0.11)	48.80	252.70 (±3.68)
10	30 million	5.38	300	4.25 (±0.04)	7.50	302.00 (±11.71)
11	50 million	5.60	300	4.95 (±0.07)	22.36	310.41 (±17.45)
12	70 million	5.75	300	4.93 (±0.09)	15.38	306.81 (±8.15)
13[2]	30 million	5.38	0	7.97 (±0.25)	NA	ND

[1]Results after 30 d of storage at 6°C are reported in terms of cell load of *K. marxianus* B0399, percentage of cell survival, and sorbic acid concentration in fermented milks. Standard deviations are reported in parentheses.

[2]For this combination, the results regarding cell load referred to 4 d of storage, cell survival percentage was not applicable (NA) and sorbic acid was not detectable (ND; less than the limit of quantification; i.e., 1.2 mg/kg).

wt/wt) were added. Finally, a suspension (3% wt/wt) containing proper amounts of *K. marxianus* B0399 and sorbic acid (added as potassium sorbate), depending on the experimental plan, was added. Fermented milk was then cooled at 10°C, portioned in 125-g plastic cups, sealed, and kept at 4°C. The obtained samples were stored at different temperatures and monitored for 30 d to evaluate the survival of *K. marxianus* B0399 and the sorbic acid concentration.

Experimental Plan. In the first part of the research, a preliminary evaluation of the effects of thermal abuses on the yeast survival was performed. Fermented milk samples were produced and inoculated with *K. marxianus* B0399 (5 log cfu/g) immediately before cooling. Two different fermented milk productions were obtained, characterized by a different sorbic acid concentration (trial A = 270 mg/kg; trial B = 150 mg/kg). The fermented milk samples were stored at 4, 6, 8, 10, and 12°C for 30 d and yeast concentration was periodically monitored by plate counting on YPD.

After this preliminary experiment, with the aim to optimize the ratio between initial yeast inoculum and sorbic acid concentration, 13 different combinations of weak acid concentration and initial *K. marxianus* B0399 inoculum were evaluated. For this purpose, fermented milk industrially produced without yeast and sorbic acid was manually inoculated; in particular, 3.75 mL of a suspension containing proper amounts of *K. marxianus* B0399 and sorbic acid was added to each cup to reach the concentrations reported in the final product in Table 1. Samples obtained (about 15 for each condition) were stored at 6°C and analyzed after 30 d.

Finally, 2 industrial productions, using the combinations that showed the best results in the previous trial, were prepared. In particular, the production defined as low (**L**) was characterized by lower levels of yeast and sorbic acid (30 million cells per serving and 150 mg/kg, respectively), whereas the high (**H**) concentrations of *K. marxianus* B0399 and sorbic acid were characterized as 70 million cells per serving and 250 mg/kg, respectively. Sample were stored at 4, 6, and 10°C and monitored for 35 d. Moreover, the effect of abuse at room temperature (24 ± 2°C) for 6 h (before storage at 6 or 10°C) was evaluated to simulate what could happen during consumer purchase and carriage.

Microbial Count of Kluyveromyces marxianus B0399 and pH Analyses. For microbiological analyses, approximately 10 g of sample were 10 fold diluted with 0.9% (wt/vol) NaCl solution and homogenized in a Lab Blender Stomacher (Seward Medical, London, UK) for 2 min. Decimal dilutions were performed and plated onto YPD agar (yeast extract 0.5%, universal peptone 1%, dextrose 2%, agar 2%; Oxoid) with 200 mg/L of chloramphenicol added. Plates were incubated at 37°C for 72 h. The pH was measured directly in the cups by using a pH-meter Basic 20 (Crison Instruments, Barcelona, Spain). Data reported are the mean of 3 determinations.

Organic Acids Determination. The concentration of organic acids was determined by an HPLC [PU-2089 Intelligent HPLC quaternary pump, UV–VIS multiwavelength detector UV 2070 Plus (Jasco Corp., Tokio, Japan)] and a manual Rheodyne injector equipped with a 20-μL loop (Rheodyne, Rohnert Park, CA). Ten

grams of each sample were added with 40 mL of 0.1 *N* sulphuric solution and the mixture was homogenized for 10 min by an Omni Mixer Homogenizer (Omni International, Warrengton, VA) and filtered through a 0.22-μm membrane filter. The separation was performed at room temperature on an Atlantis C18 column (Waters Corp., Milford, MA) 250 × 4.6 mm, 5 μm thickness with an isocratic elution during 20 min, followed by 10 min of washing, and a re-equilibration period. The mobile phase was 0.02 *M* NaH_2PO_4 adjusted to pH 2.75 with phosphoric acid and the flow rate was 0.6 mL/min. The detector was set at 210 nm.

Sorbic Acid Determination. The extraction of sorbic acid from the samples was carried out as described by Tfouni and Toledo (2002). Briefly, 5 g of sample were added with 15 mL of 2% H_3PO_4. The solution was shaken for 1 min and, subsequently, 30 mL of acetonitrile was added and the solution shaken again for 1 min. The mixture was centrifuged for 5 min at 3,000 rpm at 4°C and filtered before the analysis. The reversed phase-HPLC analysis was performed by a HP 1200 Series instrument (Agilent Technologies, Palo Alto, CA) equipped with a binary pump delivery system, a degasser, an autosampler (automatic liquid sampler), a UV-VIS detector, and an HP-mass spectrometer detector (model G1946A); integration and data elaboration were performed using Chemstation software (Hewlett Packard, Palo Alto, CA). The sorbic determination method has been adapted from the method proposed by Kamankesh et al. (2013); in particular, in the present study a new-generation column, that is, a Poroshell 120 EC-C18 30 × 50 mm, 2.7 μm thickness (Agilent Technologies), was used. All solvents were HPLC-grade and filtered with a 0.45-μm filter disk. An isocratic program was carried out using a solution of NH_4COOH (0.05 *M*, pH 4.4), acetonitrile 60:40 (vol/vol) as mobile phase. The time analysis was 4 min at a flow of 1 mL/min. The chromatograms were registered at 254 nm; the injection volume was 2.5 μL. All analyses were carried out at 25°C. Calibration curve of sorbic acid was arranged from limit of quantification (**LOQ**; 150 mg/kg) at 6 concentration levels, plotting peak area versus analyte concentration. The HPLC analysis was replicated 3 times for extracts and calibration points (n = 3).

RESULTS

Evaluation In Vitro of MIC and MFC of Sorbic Acid on K. marxianus B0399

The effect of sorbic acid on *K. marxianus* B0399 viability and growth was first assessed by determination of its MIC and MFC carried out in a culture medium (YPD). Tests were performed at pH 6.5 (optimal growth condition) and 4.2 (value similar to pH of the target product), considering an initial inoculum of 5 log cfu/mL. The results showed that MIC of sorbic acid against *K. marxianus* B0399 were 650 and 175 μg/mL at pH 6.5 and 4.2, respectively, whereas MFC values were 850 and 400 μg/mL at the same pH values.

Due to these results, in all the experiments carried out using fermented milk, the addition of sorbate, aimed to inhibit yeast growth without negative effects on its survival, never exceeded 300 mg/L. This is the maximum level allowed for flavored fermented milk products, according to European regulations (European Commission, 2011).

Repeatability and Sensitivity of Sorbic Acid Determination Method

To determine sorbic acid, a method adapted from Kamankesh et al., (2013) was used. According to the HPLC procedure adopted, the retention time for sorbic acid was 0.67 min. The sensitivity of the method was studied by defining the limits of detection (**LOD**) and LOQ for sorbic acid in standard solutions. The LOD and LOQ were, respectively, set at S/n = 3 and 10, where S/N is the signal-to noise ratio. The LOD of the method was obtained at 0.36 mg/kg and LOQ was established at 1.2 mg/kg. The linearity was obtained in the range of 1 to 150 mg/kg. The repeatability was assessed for an extract. The extract was injected 5 times on the same day (intraday precision) and for 3 consecutive days (interday precision, n = 15) and the percent relative standard deviations (**RSD**) of the peak areas (UV detection) and retention times were determined. The intraday repeatability (expressed as RSD) of the retention times was 0.26%, whereas the interday repeatability was 0.87%. The intraday repeatability (expressed as RSD) of the total peak area was 0.47%, whereas the interday repeatability was 1.03%. These values results significantly lower than the results reported by Kamankesh et al. (2013), where an intraday repeatability (expressed as RSD) of the total peak area was equal to 4.58%.

Compared with literature using other protocols, the proposed method showed a good sensitivity and repeatability and fast time of analysis; therefore, it is suitable for routine analysis in the food industry. In fact, this method, with the use of a fused core column, permits a 5-fold reduction of time analysis with respect to the method proposed by Kamankesh et al. (2013).

Preliminary Evaluation of the Effect of Sorbic Acid on K. marxianus B0399 Viability in Fermented Milk

In the first experiment, the effect of 2 sorbic acid concentrations (270 and 150 mg/kg) on *K. marxianus* B0399 viability and growth in fermented milk samples stored at different temperatures was evaluated. The evolutions of the cell load of the yeast during a 30 d storage at different temperature (4, 6, 8, 10, and 12°C) are reported in Figure 1a (trial A = 270 mg/kg of sorbic acid) and Figure 1b (trial B = 150 mg/kg of sorbic acid). In the presence of the higher sorbic acid concentrations at lower temperatures (4 and 6°C) the initial cell load remained almost constant throughout all the storage period; at higher temperatures a decrease of *K. marxianus* B0399 viability was detected starting from d 12. After 30 d, in the samples at 8, 10, and 12°C, cell load of the yeast decreased down to 3.5 to 3.7 log cfu/g. A different behavior was observed when the lower concentration of sorbic acid (150 mg/kg) was added to fermented milk (Figure 1b). In this case, the yeast cell load was constant at 4 and 6°C (as reported for higher sorbic acid amount), but it generally increased at higher temperature. In fact, about 30% of samples kept at temperature higher than 6°C showed, after 30 d of storage, an evident swelling of the package due to the accumulation of CO_2 produced by *K. marxianus* B0399. Therefore, data regarding the last sampling time, reported as means of 2 intact packages and 1 swollen, showed cell counts of about 5.5 log cfu/g with high standard deviations (Figure 1b).

Evaluation of Different Combinations of Sorbic Acid and K. marxianus B0399 in Relation to Probiotic Survival

The second part of our study aimed to find the best combination of *K. marxianus* B0399 inoculum and sorbic acid concentration, able to ensure the presence of at least 20 million viable cells (corresponding to 5.20 log cfu/g) in each fermented milk serving (a 125-g cup) for at least 30 d, avoiding package swelling due to excessive yeast growth.

For this purpose, 13 combinations of sorbic acid concentration and yeast inocula were tested. Combination 13, characterized by the presence of yeast (30 million cells per serving) without preservative, was used as a control. The results regarding cell load, percentage of cell survival, and sorbic acid concentration after 30 d of storage at 6°C are reported in Table 1. As all the samples of combination 13 (which did not contain sorbic acid) showed an evident swelling of the package after few days of storage, the data reported in Table 1 (i.e., yeast counts of 7.97 log cfu/g) referred to 4 d of storage and the products were not further sampled. None of the other fermented milks showed swelling or any other visible alteration during storage. The presence of 150 mg/kg of sorbic acid (combinations from 1 to 3) did not affect *K. marxianus* B0399 viability during storage at 6°C, independent of the initial inoculum; in fact, the yeast counts were almost constant after 30 d, and the percentage of cell survival was above 95%. The samples characterized by the presence of 200 and 250 mg/kg of sorbic acid (combinations from 4 to 9) showed a similar behavior, with percentages of cell survival ranging between 45 and 63%. When preservative concentration increased up to 300 mg/kg, a drastic negative effect on *K. marxianus* B0399 viability was observed. In fact, the percentage of surviving cells was below 23% for all the samples and, at the lower inoculum level (30 million cells per serving), it reached 7.5% of the initial cell load. As for sorbic acid, data reported in Table 1 showed that its concentration did not significantly change during storage. From these results, it was possible to identify which combinations can ensure the yeast cell load required by the company (i.e., 5.20 log cfu/g). The higher percentage of survivors were observed in all the samples characterized by the lowest sorbic acid concentration (150 mg/kg) and in the samples with 200 or 250 mg/kg of preservative, when the initial inoculum of *K. marxianus* B0399 was 50 or 70 million cells per serving. Therefore, the combination with 150 (lowest amount) and 250 mg/kg (maximum tested level that did not strongly affect yeast viability) of sorbic acid were chosen for further investigation at industrial level, and they were respectively combined with the lowest (30 million cells per serving) and the highest (70 million cells per serving) inoculum considered in the experimental plan.

Individuation of the Optimal Conditions for the Commercial Product

In the last part of the study, industrial-scale productions (batches of 4,000 L of milk) were manufactured using the 2 combinations that gave the most promising results at laboratory scale (as previously described). Samples of the 2 productions were stored at 4 (control), 6, and 10°C for 35 d (i.e., 5 d after the established shelf-life). For the samples at 6 and 10°C, the effect of abuse at room temperature (24 ± 2°C) for 6 h before refrigeration was also investigated. The results regarding *K. marxianus* B0399 cell load during storage are reported in Figure 2a for the production characterized by lower concentrations of yeast and sorbic acid (batch L = 30 million cells and 150 mg/kg, respectively), and in Figure 2b for the production with higher levels of *K. marxianus* B0399 and sorbic acid (batch H = 70

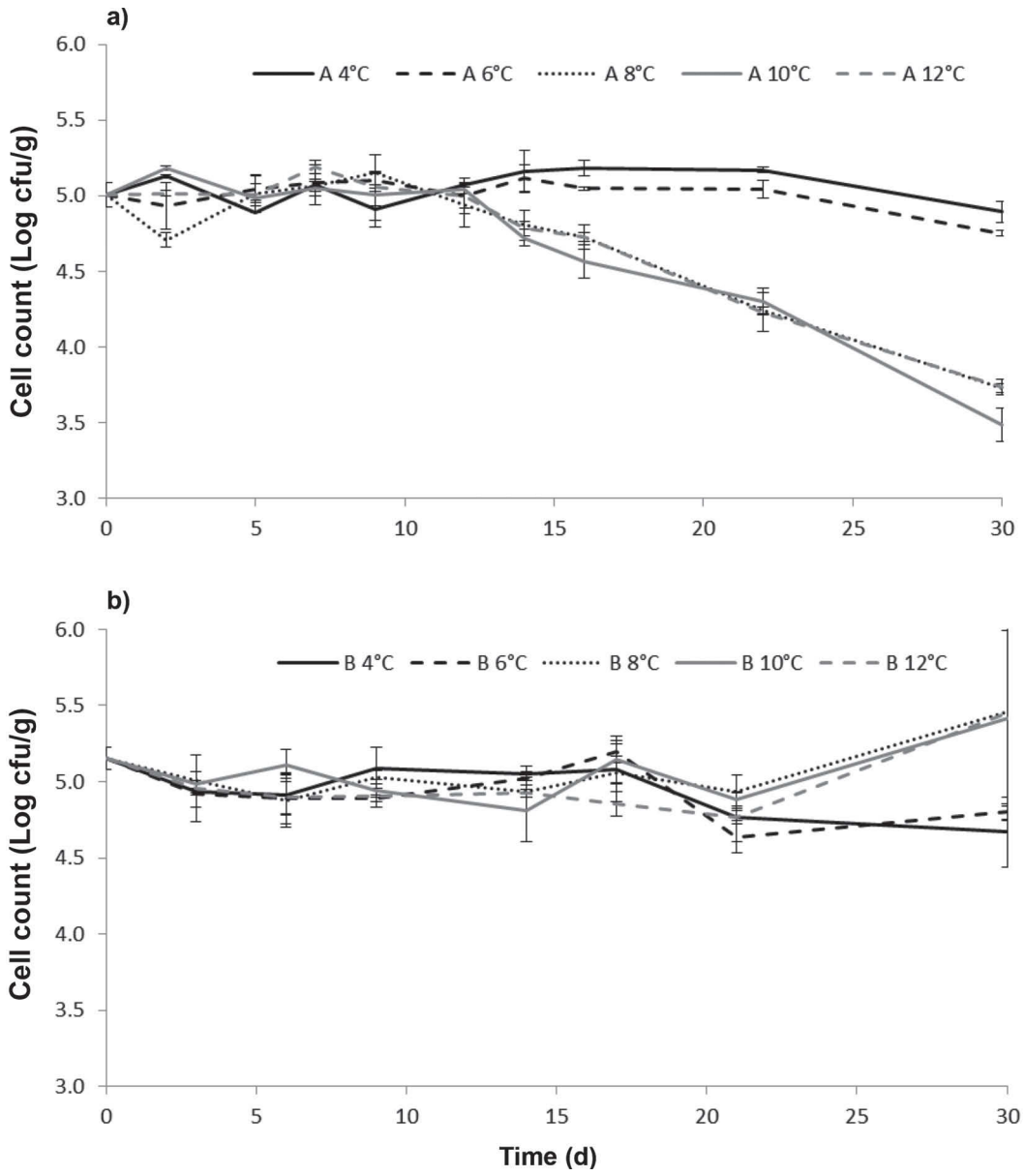

Figure 1. Evolution of cell load of *Kluyveromyces marxianus* B0399 (initial inoculum level: about 5 log cfu/g) in fermented milks with addition of (a) 270 or (b) 150 mg/kg of sorbic acid and stored at different temperatures for 30 d. Full black line = storage at 4°C; dashed black line = storage at 6°C; dotted black line = storage at 8°C; full gray line = storage at 10°C; dashed gray line = storage at 12°C. Error bars indicate SD.

million cells and 250 mg/kg). As seen in the figures, the scale up of production at industrial level determined, in the final product, cell loads slightly lower than that expected by the experimental plan. In fact, the initial concentration of *K. marxianus* B0399 in fermented milks of batch L was 5.31 log cfu/g, instead of the targeted 5.38 log cfu/g (corresponding to 30 million cells per serving). Similarly, samples of batch H showed yeast counts of 5.62 log cfu/g, instead of 5.75 log cfu/g (corresponding to 70 million cells per serving).

Regarding the effect of storage temperature, in fermented milks starting with a cell load of 5.31 log cfu/g (batch L), this initial value remained quite constant until 35 d of storage in the control samples (4°C) and at 6°C, independent of the presence of abuse at room temperature (Figure 2a). On the contrary, the conservation at 10°C induced a strong decrease of *K. marxianus* B0399 viability starting from d 21 of storage. In fact, these samples were characterized, after 30 d of storage (established shelf-life of the fermented milks), by *K.*

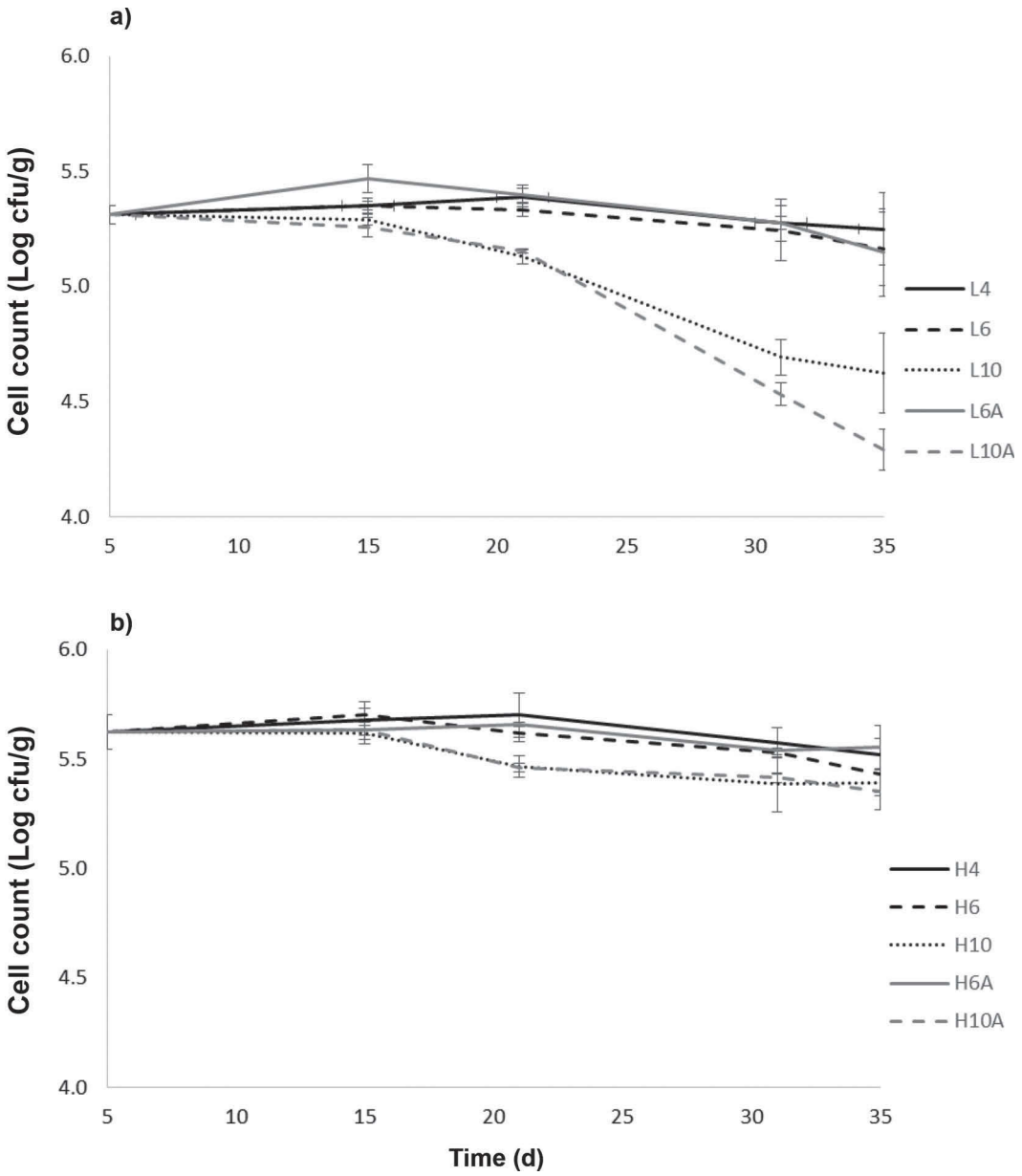

Figure 2. Evolution of *Kluyveromyces marxianus* B0399 cell load during storage in the industrial productions (a = batch L; b = batch H) characterized by 2 different concentrations of yeast and sorbic acid (i.e., L = 30 million yeast cells and 150 mg/kg of sorbic acid; H = 70 million yeast cells and 250 mg/kg of sorbic acid). Full black line = storage at 4°C; dashed black line = storage at 6°C; dotted black line = storage at 10°C; full gray line = storage at 6°C after thermal abuse; dashed gray line = storage at 10°C after thermal abuse. Error bars indicate SD.

marxianus B0399 counts of about 4.5 to 4.7 log cfu/g. When storage was prolonged until 35 d, a further decrease of cell load was observed, especially in products stored at 10°C after abuse at room temperature for 6 h, in which the final *K. marxianus* B0399 count was 4.3 log cfu/g.

Fermented milk containing the higher concentrations of yeast and sorbic acid (batch H) showed a different behavior and the effect of temperature was less evident (Figure 2b). In fact, in this case microbial counts of samples at 4 and 6°C also remained constant but, despite what observed at lower inoculum, higher storage temperature (10°C) only slightly affected *K. marxianus* B0399 loss of viability (0.1 log cfu/g) and the effect of a 6 h abuse at room temperature was negligible.

The data regarding pH, organic acids, and sorbic acid at the beginning and at the end of storage are reported in Table 2. The pH immediately after packaging was 3.94 and 3.97 for batch L and batch H, respectively, without significant difference among the productions.

Table 2. pH values, amount of organic acids (expressed as g/kg) and of sorbic acid (expressed as mg/kg) in fermented milks derived from low (L) and high (H) production; SD are reported in parentheses

Time	Sample[1]	pH	Organic acids (g/kg)				Sorbic acid (mg/kg)
			Lactic acid	Acetic acid	Citric acid	Malic acid	
d 1	L4	3.94 (±0.03)	6.61 (±0.18)	0.16 (±0.02)	0.94 (±0.03)	0.47 (±0.02)	149.4 (±1.5)
	H4	3.97 (±0.04)	6.51 (±0.21)	0.18 (±0.03)	0.94 (±0.04)	0.47 (±0.03)	234.0 (±2.1)
d 35	L4	3.71 (±0.03)	7.85 (±0.12)	0.16 (±0.05)	0.96 (±0.03)	0.44 (±0.02)	148.0 (±1.7)
	L6	3.70 (±0.02)	8.21 (±0.24)	0.16 (±0.02)	0.96 (±0.05)	0.42 (±0.03)	152.3 (±2.2)
	L10	3.64 (±0.05)	8.56 (±0.23)	0.31 (±0.03)	0.89 (±0.02)	0.45 (±0.05)	151.6 (±2.6)
	L6A	3.67 (±0.04)	8.25 (±0.13)	0.32 (±0.02)	0.88 (±0.04)	0.47 (±0.04)	146.0 (±1.8)
	L10A	3.63 (±0.02)	8.53 (±0.29)	0.25 (±0.04)	0.89 (±0.03)	0.44 (±0.02)	153.9 (±2.1)
	H4	3.73 (±0.05)	8.78 (±0.31)	0.26 (±0.04)	1.01 (±0.06)	0.44 (±0.03)	238.1 (±3.6)
	H6	3.72 (±0.03)	8.48 (±0.15)	0.23 (±0.02)	0.92 (±0.04)	0.44 (±0.02)	240.6 (±4.0)
	H10	3.64 (±0.02)	10.01 (±0.33)	0.33 (±0.03)	0.89 (±0.03)	0.45 (±0.04)	238.0 (±2.4)
	H6A	3.74 (±0.04)	8.17 (±0.17)	0.21 (±0.04)	0.86 (±0.05)	0.44 (±0.02)	229.0 (±6.2)
	H10A	3.65 (±0.04)	9.75 (±0.27)	0.28 (±0.02)	0.87 (±0.05)	0.43 (±0.03)	235.3 (±4.6)

[1]The first letter refers to the inoculum level (L = 30 million yeast cells and 150 mg/kg of sorbic acid; H = 70 million yeast cells and 250 mg/kg of sorbic acid); the number is the storage temperature (expressed in °C); the presence of the letter A indicates that the product was subjected to thermal abuse (6 h at room temperature).

In all the samples, the initial values slightly decreased during storage and, after 35 d, they were lower by about 0.1 to 0.3 pH units, depending on the refrigeration conditions.

Lactic acid was present at the beginning of storage at a concentration of about 6.55 g/kg. Its amount increased during storage in all the samples, depending on the initial inoculum and the conservation temperature. In fact, in fermented milk of batch L kept at 4°C for 35 d, lactic acid concentration increased by about 1.2 g/kg. This increment was double in samples with a higher initial inoculum (batch H) and stored at the same temperature, in which lactic acid reached about 8.75 g/kg at the end of storage. The accumulation of lactic acid during storage was more evident in samples stored at 6 and 10°C, especially when the initial inoculum was higher (batch H). In fact, in these samples at 10°C, lactic acid was detected at a concentration of about 10 g/kg without significant differences in relation to the presence of abuse at room temperature before storage.

The concentrations of acetic acid at first sampling time were about 0.16 and 0.18 g/kg for batch L and H, respectively. After 35 d of conservation, the amount of this organic acid did not significantly change in the samples of batch L stored at 4 and 6°C, whereas it slightly increased in all the other fermented milks.

The slightly increasing trend observed for these 2 acids can be explained by the activities of the lactic acid bacteria used for fermented milk preparation. In fact, after 35 d of storage, the residual viable cells were higher than 6.5 log cfu/g for all the bacterial species used (i.e., *Streptococcus thermophilus*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, and *Bifidobacterium animalis* ssp. *lactis*; data not shown).

The initial amount of citric acid was about 0.94 g/kg, mainly derived from the puree added. After 35 d, the amount of this acid slightly decreased in the samples stored at 10°C and in the samples at 6°C subjected to thermal abuse. Malic acid, also derived from fruit puree, was detected in all the samples at level of about 0.4 g/kg, without significant difference in relation to time and temperature of storage.

With regard to sorbic acid, its initial concentrations were expected to be, according to the experimental plan, 150 and 250 mg/kg for batch L and batch H, respectively. Data reported in Table 2 showed that, in batch L, the initial concentration of sorbic acid complied with the expected value (150 mg/kg); whereas, in the fermented milk deriving from batch H, scaling up resulted in a lower concentration of sorbic acid in the final product (234 instead of the expected 250 mg/kg). These initial values remained almost stable throughout conservation, independent of storage temperature.

DISCUSSION

Foods with added functional or probiotic microorganisms not only need to be safe at the end of shelf-life, but must also keep their functional characteristics throughout the same period (Karaolis et al., 2013). In particular, the viability of the functional microorganisms should remain above a threshold level, defined as the minimum intake necessary for promoting their health properties, for the entire product commercialization time. Consequently, the crucial problem for probiotic bacteria in food is to maintain the cell survival as high as possible.

If functional yeasts are added to fermented milks, the objective is double; in fact, besides the maintenance of yeast viability, the addition of a preservative can also be necessary to avoid their proliferation, which could result in the swelling of the fermented milk package. The main strategy to reach this goal is the control of the cold chain at 0 to 4°C. Unfortunately, this condition is often not rigorously applied, especially during transport, in the chilled cabinets of the retail markets and during domestic storage (Jacxsens et al., 2002). For this reason, the effects of fluctuating temperature and thermal abuse should be considered in the preparation of functional refrigerated fermented milks.

Whereas much research exists about probiotic bacteria (Ross et al., 2002; Shah, 2004), little information is available about the technological aptitude of functional yeasts. The yeast *Saccharomyces boulardii* has been used to contrast several gastrointestinal diseases and has been included in functional food formulations (Martins et al., 2005; Karaolis et al., 2013; Khatri et al., 2013). The identification of functional properties in *K. marxianus* B0399 (Maccaferri et al., 2012a) opened new perspectives for the technological exploitation of the potential of this strain added to fermented milk.

In the formulation of a new industrial product containing the functional yeast *K. marxianus* B0399, the sorbic acid concentration used was a critic al point because it had to ensure the absence of growth without compromising yeast cell viability. Sorbic acid is a weak acid with a pH-dependent antimicrobial activity, and is particularly active against molds and yeasts. Another important factor influencing the antimicrobial activity of this acid is the yeast concentration (Stopforth et al., 2005). The MIC determined in vitro in these trials is in agreement with the data reported by Praphailong and Fleet (1997), but it is lower than that found by Goretti et al. (2009). Many bacteria, among them lactic acid bacteria, can degrade this weak acid, producing geranium-like off-odors and causing the loss of antimicrobial activity (Sofos, 1989). In any case, in the fermented milk we studied, sorbate was not degraded during storage.

Interestingly, the higher storage temperatures caused 2 opposite effects on *K. marxianus* B0399. At the higher sorbate concentrations (270 mg/kg) the viability of the yeast drastically decreased in the second part of storage, whereas this did not happen under the same conditions at lower temperatures. In other words, the enhancement of yeast metabolism due to the increased temperature made it more susceptible to the antifungal activity of sorbate. By contrast, the same temperature shift in the presence of 150 mg/kg of the weak acid caused a proliferation of the yeast with a visible spoilage of the fermented milk. This opposite effect underlines the need to find a precise equilibrium between sorbate amount and *K. marxianus* B0399 inoculum to guarantee the desired commercial shelf-life in relation to the organoleptic features and functional properties.

Kluyveromyces marxianus is a Crabtree-negative yeast, as no ethanol production was observed after a glucose pulse was applied to respiring cells, in contrast to what is commonly observed with *S. cerevisiae.* Thus, it shows a fermentative metabolism only in the presence of low oxygen concentrations but not in the absence of oxygen (Fonseca et al., 2008). For this reason, an alternative strategy for avoiding undesirable spoilage of the fermented milks in which this yeast is added could be the exclusion of this gas from the headspace of the packaged product. Nevertheless, further experiments to evaluate the effect of oxygen absence on the loss of viability of the yeast are needed before adopting such a strategy.

CONCLUSIONS

This work demonstrates the possibility to industrially produce fermented milk containing the functional yeast *K. marxianus* B0399, whose viability is maintained throughout a commercialization time of 30 d, even in the presence of temperature abuses (during transport, retail market storage, and domestic conservation). This was achieved by using a suitable sorbic acid concentration in relation to the initial yeast cell load. This fragile equilibrium has to be guaranteed by a precise formulation during fermented milk manufacturing. Further strategies can be exploited based on some peculiar metabolic characteristics of this strain.

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